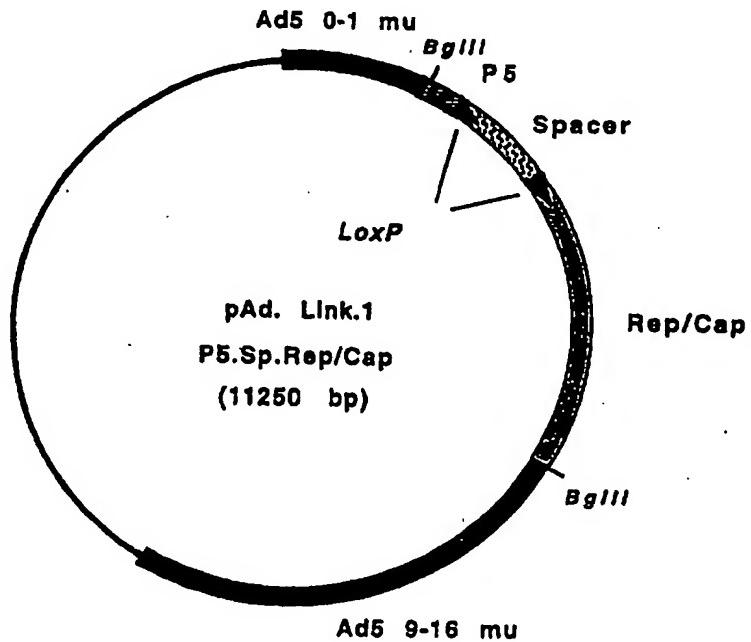


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(54) Title: METHODS USING CRE-LOX FOR PRODUCTION OF RECOMBINANT ADENO-ASSOCIATED VIRUSES



(57) Abstract

Methods for efficient production of recombinant AAV are described. In one aspect, three vectors are introduced into a host cell. A first vector directs expression of cre recombinase, a second vector contains a promoter, a spacer sequence flanked by loxP sites and rep/cap, and a third vector contains a minigene containing a transgene and regulatory sequences flanked by AAV ITRs. In another aspect, the host cell stably or inducibly expresses cre recombinase and two vectors carrying the other elements of the system are introduced into the host cell.

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METHODS USING CRE-LOX FOR PRODUCTION OF
RECOMBINANT ADENO-ASSOCIATED VIRUSES

Field of the Invention

This invention relates generally to production
5 methods for recombinant viruses, and more specifically,
to methods of producing recombinant adeno-associated
viruses.

Background of the Invention

Adeno-associated virus (AAV) is a replication-
10 deficient parvovirus, the genome of which is about 4.6 kb
in length, including 145 nucleotide inverted terminal
repeats (ITRs). Two open reading frames encode a series
of rep and cap polypeptides. Rep polypeptides (rep78,
rep68, rep62 and rep40) are involved in replication,
15 rescue and integration of the AAV genome. The cap
proteins (VP1, VP2 and VP3) form the virion capsid.
Flanking the rep and cap open reading frames at the 5'
and 3' ends are 145 bp inverted terminal repeats (ITRs),
the first 125 bp of which are capable of forming Y- or T-
20 shaped duplex structures. Of importance for the
development of AAV vectors, the entire rep and cap
domains can be excised and replaced with a therapeutic or
reporter transgene [B. J. Carter, in "Handbook of
Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168
25 (1990)]. It has been shown that the ITRs represent the
minimal sequence required for replication, rescue,
packaging, and integration of the AAV genome.

When this nonpathogenic human virus infects a
human cell, the viral genome integrates into chromosome
30 19 resulting in latent infection of the cell. Production
of infectious virus and replication of the virus does not
occur unless the cell is coinfecte with a lytic helper
virus, such as adenovirus or herpesvirus. Upon infection
with a helper virus, the AAV provirus is rescued and
35 amplified, and both AAV and helper virus are produced.

The infecting parental ssDNA is expanded to duplex replicating form (RF) DNAs in a *rep* dependent manner. The rescued AAV genomes are packaged into preformed protein capsids (icosahedral symmetry approximately 20 nm in diameter) and released as infectious virions that have packaged either + or - ss DNA genomes following cell lysis.

AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells. Various groups have studied the potential use of AAV in the treatment of disease states. Progress towards establishing AAV as a transducing vector for gene therapy has been slow for a variety of reasons. While the ability of AAV to integrate in quiescent cells is important in terms of long term expression of a potential transducing gene, the tendency of the integrated provirus to preferentially target only specific sites in chromosome 19 reduces its usefulness.

However, an obstacle to the use of AAV for delivery of DNA is lack of highly efficient schemes for encapsidation of recombinant genomes and production of infectious virions. See, R. Kotin, Hum. Gene Ther., 5:793-801 (1994)]. One such method involves transfecting the rAAV genome into host cells followed by co-infection with wild-type AAV and adenovirus. However, this method leads to unacceptably high levels of wild-type AAV. Incubation of cells with rAAV in the absence of contaminating wild-type AAV or helper adenovirus is associated with little recombinant gene expression. In the absence of *rep*, integration is inefficient and not directed to chromosome 19.

A widely recognized means for manufacturing transducing AAV virions entails co-transfection with two different, yet complementing plasmids. One of these contains the therapeutic or reporter transgene sandwiched

between the two *cis* acting AAV ITRs. The AAV components that are needed for rescue and subsequent packaging of progeny recombinant genomes are provided in *trans* by a second plasmid encoding the viral open reading frames for 5 *rep* and *cap* proteins. Overexpression of *Rep* proteins have some inhibitory effects on adenovirus and cell growth [J. Li et al, *J. Virol.*, 71:5236-5243 (1997)]. This toxicity has been the major source of difficulty in providing these genes in *trans* for the construction of a 10 useful rAAV gene therapy vector.

There remains a need in the art for the methods permitting the efficient production of AAV and recombinant AAV viruses for use as vectors for somatic gene therapy.

15 Summary of the Invention

The present invention provides methods which permit efficient production of rAAV, which overcome the difficulties faced by the prior art. This method is particularly desirable for production of recombinant AAV 20 vectors useful in gene therapy. The method involves providing a host cell with

- (a) a *cre* transgene, which permits splicing out of the *rep* and *cap* gene inhibitory sequences that when removed lead to activation of *rep* and *cap*;
- 25 (b) the AAV *rep* and *cap* genes, 5' to these genes is a spacer which is flanked by lox sites;
- (c) a minigene comprising a therapeutic transgene flanked by AAV inverse terminal repeats (ITRs); and
- 30 (d) adenovirus or herpesvirus helper functions.

Thus, in one aspect, the invention provides a method for producing a rAAV which comprises introducing into a host cell a first vector containing the *cre*

transgene under regulatory control of sequences which express the gene product thereof *in vitro*, a second vector containing a spacer flanked by lox sites, which is 5' to the rep and cap genes, and a third vector 5 containing a therapeutic transgene flanked by AAV ITRs. These vectors may be plasmids or recombinant viruses. One of the vectors can be a recombinant adenovirus or herpesvirus, which can provide to the host cell the essential viral helper functions to produce a rAAV 10 particle. However, if all the vectors are plasmids, the cell must also be infected with the desired helper virus. The cell is then cultured under conditions permitting production of the cre recombinase. The recombinase causes deletion of the spacer flanked by lox sites 15 upstream of the rep/cap genes. Removal of the spacer allows the rep and cap genes to be expressed, which in turn allows packaging of the therapeutic transgene flanked by AAV ITRs. The rAAV is harvested thereafter.

In another aspect, the invention provides a 20 method wherein a host cell expressing cre recombinase is co-transfected with a vector carrying a spacer flanked by lox sites 5' to the rep and cap genes, and a vector containing the therapeutic minigene above. With the provision of helper functions by a means described herein, the cell is then cultured under appropriate 25 conditions. When cultured, the cre recombinase causes deletion of the spacer thus activating expression of rep/cap, resulting in the rAAV as described above.

In yet another aspect, the present invention 30 provides rAAV vectors produced by the methods of the invention.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a schematic illustration of a 1600 bp DNA fragment containing green fluorescent protein (GFP) cDNA, an intron and a polyadenylation (pA or polyA) signal useful as a spacer in a vector of the invention.

Fig. 2 is a schematic illustration of a 1000 bp DNA fragment containing the gene encoding neomycin resistance (neo^R) and a polyA useful as a spacer.

Fig. 3 illustrates a plasmid pG.CMV.nls.CRE, useful for transfection of human embryonic kidney 293 cells in the method of the invention.

Fig. 4 illustrates a plasmid pAd.P5.Sp.Rep/Cap, useful in the method of the invention.

Fig. 5 illustrates the construction of the recombinant adenovirus, Ad.CMV.NLS-CRE, useful in the method of the invention.

Fig. 6A illustrates the structure of the Ad.CAG.Sp.LacZ virus.

Fig. 6B provides the Southern blot analysis of genomic DNA isolated from 293 cells infected with the LacZ virus at a m.o.i. of 1 and cut with *NotI*. The 1000 bp ^{32}P -NEO spacer was used as a probe. After the digestion with *NotI* a 6200 bp restriction fragment (without cre-mediated recombination) and/or a 5200 bp restriction fragment (with cre-mediated recombination) can be detected.

Fig. 6C provides the Southern blot analysis of genomic DNA isolated from 293 cells infected with the LacZ virus at a m.o.i. of 10 and cut with *NotI*. The 1000 bp ^{32}P -NEO spacer was used as a probe. After the digestion with *NotI* a 6200 bp restriction fragment (without cre-mediated recombination) and/or a 5200 bp restriction fragment (with cre-mediated recombination) can be detected.

Fig. 6D provides the Southern blot analysis genomic DNA isolated from 293 cells infected with the LacZ virus at a m.o.i. of 100 and cut with *NotI*. The 1000 bp ^{32}P -NEO spacer was used as a probe. After the 5 digestion with *NotI* a 6200 bp restriction fragment (without cre-mediated recombination) and/or a 5200 bp restriction fragment (with cre-mediated recombination) can be detected.

Fig. 7 illustrates the structure of the 10 Ad.Tre.CMV.GFP.Rep/Cap virus.

Detailed Description of the Invention

The invention provides a method for rAAV production using the cre-lox system, which overcomes the difficulties previously experienced in providing 15 efficient production systems for recombinant AAV. The method of this invention produces rAAV carrying therapeutic transgenes, which are particularly useful in gene therapy applications.

In summary, the method involves culturing a 20 selected host cell which contains
(a) a cre transgene
(b) the AAV rep and cap genes, 5' to these genes is a spacer flanked by lox sites;
(c) a minigene comprising a therapeutic 25 transgene flanked by AAV ITRs; and
(d) adenovirus or herpesvirus helper functions.

The use of the term "vector" throughout this specification refers to either plasmid or viral vectors, 30 which permit the desired components to be transferred to the host cell via transfection or infection. By the term "host cell" is meant any mammalian cell which is capable of functioning as an adenovirus packaging cell, i.e., expresses any adenovirus proteins essential to the

production of AAV, such as HEK 293 cells and other packaging cells. By the term "minigene" is meant the sequences providing a therapeutic transgene in operative association with regulatory sequences directing expression thereof in the host cell and flanked by AAV ITRs. The term "transgene" means a heterologous gene inserted into a vector.

Desirably, components (a), (b) and (c) may be carried on separate plasmid sequences, or carried as a transgene in a recombinant virus. Alternatively, the cre protein may be expressed by the selected host cell, therefor not requiring transfection by a vector. For each of these components, recombinant adenoviruses are currently preferred. However, using the information provided herein and known techniques, one of skill in the art could readily construct a different recombinant virus (i.e., non-adenovirus) or a plasmid vector which is capable of driving expression of the selected component in the host cell. For example, although less preferred because of their inability to infect non-dividing cells, vectors carrying the required elements of this system, e.g., the cre recombinase, may be readily constructed using e.g., retroviruses or baculoviruses. Therefore, this invention is not limited by the virus or plasmid selected for purposes of introducing the cre recombinase, rep/cap, or minigene into the host cell.

Desirably, however, at least one of the vectors is a recombinant virus which also supplies the helper functions (d) to the cell. Alternatively, the helper functions may be supplied by co-infecting the cell with a helper virus, i.e., adenovirus or herpesvirus, in a conventional manner. The resulting rAAV containing the minigene may be isolated therefrom.

A. The Cre Transgene

The cre protein is a recombinase isolated from bacteriophage P1 which recognizes a specific sequence of 34 bp (*loxP*). Recombination between two *loxP* sites (catalyzed by the cre protein) causes, in certain cases, the loss of sequences flanked by these sites [for a review see N. Kilby et al, *Trends Genet.*, 9:413-421 (1993)]. The sequences of cre are provided in N. Sternberg et al, *J. Mol. Biol.*, 187:197-212 (1986) and may alternatively be obtained from other commercial and academic sources. The expression of the cre protein in the cell is essential to the method of this invention.

Without wishing to be bound by theory, the inventors believe that the expression of cre recombinase in the host cell permits the deletion of the "spacer" DNA sequence residing between the promoter and rep/cap genes in the second vector. This deletion of rep and cap gene inhibitory sequences, allows expression and activation of the rep and cap proteins and resulting in the replication and packaging of the AAV genome.

The cre protein may be provided in two alternative ways. The gene encoding the protein may be a separate component transfected into the desired host cell. Alternatively, the host cell selected for expression of the rAAV may express the cre protein constitutively or under an inducible promoter.

B. Triple Infection/Transfection Method

In one embodiment of the present invention, the method employs three vectors, i.e., recombinant viruses or plasmids, to infect/transfect a selected host cell for production of a rAAV. A first vector comprises the cre transgene operatively linked to expression control sequences. A second vector comprises the AAV rep and cap genes downstream of a spacer sequence which is flanked by *lox* sites and which itself is

downstream of expression control sequences. A third vector comprises the therapeutic minigene, i.e., a transgene flanked by AAV ITRs and regulatory sequences. Suitable techniques for introducing these vectors into 5 the host cell are discussed below and are known to those of skill in the art. When all vectors are present in a cell and the cell is provided with helper functions, the rAAV is efficiently produced.

10 1. First Vector

As stated above, in a preferred embodiment, a first vector is a recombinant replication-defective adenovirus containing the cre transgene operatively linked to expression control sequences in the site of adenovirus E1 deletion, e.g., Ad.CMV.NLS-CRE. 15 See Fig. 5. Preferably, as in the examples below, the cre gene is operably linked to a suitable nuclear localization signal (NLS). A suitable NLS is a short sequence, i.e., in the range of about 21 bp, and may be readily synthesized using conventional techniques, or 20 engineered onto the vector by including the NLS sequences in a PCR primer. As described in detail in Example 1 below, the cre gene and a nuclear localization signal (NLS) are obtained from a previously described plasmid.

Desirably, the cre gene is under the 25 control of a cytomegalovirus (CMV) immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. However, other suitable promoters may be readily selected by one of skill in the art. Useful promoters may be constitutive promoters or 30 regulated (inducible) promoters, which will enable control of the amount of the cre gene product to be expressed. For example, another suitable promoter includes, without limitation, the Rous sarcoma virus LTR promoter/enhancer. Still other promoter/enhancer 35 sequences may be selected by one of skill in the art.

In addition, the recombinant virus also includes conventional regulatory elements necessary to drive expression of the cre recombinase in a cell transfected with the vector. Such regulatory elements 5 are known to those of skill in the art, including without limitation, polyA sequences, origins of replication, etc.

2. Second Vector

Another, "second", vector useful in this embodiment of the method is described in Example 2 10 as Ad.sp.Rep/Cap. It contains the AAV rep and cap genes downstream of a spacer sequence which is flanked by lox sites and which itself is downstream of expression control sequences.

The AAV rep and cap sequences are 15 obtained by conventional means. Preferably, the promoter is the AAV P5 promoter. However, one of skill in the art may readily substitute other suitable promoters. Examples of such promoters are discussed above in connection with the first vector.

20 The spacer is an intervening DNA sequence (STOP) between the promoter and the gene. It is flanked by loxP sites and contains multiple translational start and stop codons. The spacer is designed to permit use of a "Recombination-Activated Gene Expression (RAGE)" 25 strategy [B. Sauer, Methods Enzymol., 225:890-900 (1993)]. Such a strategy controls the expression of a given gene (in this case, rep/cap). The spacer must be excised by expression of the cre protein of the first vector and its interaction with the lox sequences to 30 express rep/cap.

Currently, there are two particularly preferred spacers. These spacers include a 1600 bp DNA fragment containing the GFP cDNA, an intron and a polyadenylation signal (Fig. 1) which was derived from a

commercial plasmid (Clontech) as described below. A second preferred spacer is a 1300 bp fragment containing translational start and stop sequences obtained as a 1.3 kbp ScaI-SmaI fragment of pBS64 as described [M. Anton 5 and F. Graham, J. Virol., **69**:4600-4606 (1995)]. Another desirable spacer is a 1000 bp fragment containing the neomycin resistance coding sequence and a polyadenylation signal [Y. Kanegae et al, Nucl. Acids Res., **23**:3816-3821 (1995)] (see, Fig. 2).

10 Using the information provided herein, one of skill in the art may select and design other suitable spacers, taking into consideration such factors as length, the presence of at least one set of translational start and stop signals, and optionally, the presence of 15 polyadenylation sites. These spacers may contain genes, which typically incorporate the latter two elements (i.e., the start/stop and polyA sites). Desirably, to reduce the possibility of recombination, the spacer is less than 2 kbp in length. However, the invention is not 20 so limited.

As stated above, the spacer is flanked by loxP sites, which are recognized by the cre protein and participate in the deletion of the spacer. The sequences of loxP are publicly available from a 25 variety of sources [R. H. Hoess and K. Abremski, Proc. Natl. Acad. Sci., **81**: 1026-1029 (1984)]. Upon selection of a suitable spacer and making use of known techniques, one can readily engineer loxP sites onto the ends of the spacer sequence for use in the method of the invention.

30 In addition, the recombinant virus which carries the rep/cap genes and the spacer, also includes conventional regulatory elements necessary to drive expression of rep and cap in a cell transfected with the recombinant virus, following excision of the

loxP-flanked spacer by the cre recombinase. Such regulatory elements are known to those of skill in the art.

3. Third Vector

5 The third vector contains a minigene, which is defined as a sequence which comprises a suitable transgene, a promoter, and other regulatory elements necessary for expression of the transgene, all flanked by AAV ITRs. In the examples below, where the third vector
10 carries the LacZ gene, the presence of rAAV is detected by assays for beta-galactosidase activity. However, desirably, the third vector carries a therapeutic gene which can be delivered to an animal via the rAAV produced by this method.

15 The AAV sequences employed are preferably the *cis*-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. The ITR sequences are about 143 bp
20 in length. Preferably, substantially the entire sequences encoding the ITRs are used in the vectors, although some degree of minor modification of these sequences is expected to be permissible for this use. The ability to modify these ITR sequences is within the
25 skill of the art. [See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989); Carter et al, cited above; and K. Fisher et al., *J. Virol.*, 70:520-532 (1996)].

30 The AAV ITR sequences may be obtained from any known AAV, including presently identified human AAV types. Similarly, AAVs known to infect other animals may also be employed in the vector constructs of this invention. The selection of the AAV is not anticipated

to limit the following invention. A variety of AAV strains, types 1-4, are available from the American Type Culture Collection or available by request from a variety of commercial and institutional sources. In the 5 following exemplary embodiment an AAV-2 is used for convenience.

The 5' and 3' AAV ITR sequences flank the selected transgene sequence and associated regulatory elements. The transgene sequence of the vector is a 10 nucleic acid sequence heterologous to the AAV sequence, which encodes a polypeptide or protein of interest. The composition of the transgene sequence will depend upon the use to which the resulting vector will be put. For example, one type of transgene sequence includes a 15 reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include without limitation an *E. coli* beta-galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene and a green fluorescent protein gene. These sequences, when 20 associated with regulatory elements which drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc.

A more preferred type of transgene 25 sequence is a therapeutic gene which expresses a desired gene product in a host cell. These therapeutic nucleic acid sequences typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited 30 genetic defect or treat an epigenetic disorder or disease. The selection of the transgene sequence is not a limitation of this invention.

In addition to the major elements identified above, the minigene also includes conventional 35 regulatory elements necessary to drive expression of the

transgene in a cell transfected with this vector. Thus, the minigene comprises a selected promoter which is linked to the transgene and located within the transgene between the AAV ITR sequences.

5 Selection of the promoter used to drive expression of the transgene is a routine matter and is not a limitation of the vector. Useful promoters include those which are discussed above in connection with the first vector component.

10 The minigene also desirably contains heterologous nucleic acid sequences including sequences providing signals required for efficient polyadenylation of the transcript and introns with functional splice donor and acceptor sites. A common poly-A sequence which
15 is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted following the transgene sequences and before the 3' AAV ITR sequence. A common intron sequence is also derived from SV-40, and is
20 referred to as the SV-40 T intron sequence. A minigene of the present invention may also contain such an intron, desirably located between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional and many such sequences
25 are available [see, e.g., Sambrook et al, and references cited therein].

The rAAV vector containing the minigene may be carried on a plasmid backbone and used to transfect a selected host cell or may be flanked by viral
30 sequences (e.g., adenoviral sequences) which permit it to infect the selected host cell. Suitable Ad/AAV recombinant viruses may be produced in accordance with known techniques. See, e.g., International patent applications WO96/13598, published May 9, 1996;

WO95/23867 published Sept. 8, 1995, and WO 95/06743 published March 9, 1995, which are incorporated by reference herein.

5 C. Host Cell/Double Infection or Transfection System

In another embodiment of the method of this invention, a packaging cell line is constructed which expresses the cre recombinase. According to this aspect of the method, this cell line expressing the cre recombinase can be substituted for the vector or plasmid bearing the cre gene, as described above. Thus, only the second and third vectors described above are subsequently introduced into the cell.

15 An exemplary suitable cre expressing cell line has been generated using the vector illustrated in Fig. 3. Generation of this cell line is described in detail in Example 4 below. However, the present invention is not limited to these constructs. Given the information provided herein, one of skill in the art can 20 readily generate another plasmid containing a suitable selectable marker (e.g., neo^R). Such a plasmid may then be used for the generation of a cre recombinase-expressing cell line according to the invention.

Having obtained such a cre-expressing cell 25 line, this cell line can be infected (or transfected) with the vector containing the rep/cap genes and the vector containing the minigene described above.

D. Production of Vectors and rAAV

Assembly of the selected DNA sequences 30 contained within each of the vectors described above utilize conventional techniques. Such techniques include cDNA cloning such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the adenovirus, AAV genome combined with

polymerase chain reaction, and any other suitable methods which provide the desired nucleotide sequence.

Whether using the three vector system, or the cre-expressing host cell and two vectors,

- 5 introduction of the vectors into the host cell is accomplished using known techniques. Where appropriate, standard transfection and co-transfection techniques are employed, e.g., CaPO₄ transfection techniques using the complementation human embryonic kidney (HEK) 293 cell
10 line (a human kidney cell line containing a functional adenovirus E1a gene which provides a transacting E1a protein). Other conventional methods employed in this invention include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of
15 measuring signal generation, and the like.

Following infection/transfection, the host cell is then cultured under standard conditions, to enable production of the rAAV. See, e.g., F. L. Graham and L. Prevec, Methods Mol. Biol., 1:109-128 (1991).

- 20 Desirably, once the rAAV is identified by conventional means, it may be recovered using standard techniques and purified.

- The following examples illustrate the preferred methods of the invention. These examples are
25 illustrative only and are not intended to limit the scope of the invention.

Example 1 - Construction of Ad.CMV.NLS-CRE

- The construction of a recombinant adenovirus containing a nuclear localization signal and the cre gene
30 under control of a cytomegalovirus promoter is described below, with reference to Fig. 5.

The nls-Cre cDNA was isolated from the plasmid pexCANCRE [Y. Kanegae et al, Nucl. Acids Res., 23:3816-3821 (1995)] by digestion with SfcII and PacI and then

blunt ended with Klenow and T4 DNA polymerase. The NLS-Cre fragment was then cloned into the EcoRV site of the plasmid pAd.CMV.Link (a plasmid containing the human Ad5 sequences, map units 0 to 16, which is deleted of Ela and 5 E1b as described in X. Ye et al, J. Biol. Chem., 271:3639-3646 (1996)). The orientation and presence of the nuclear localization signal in the resulting plasmid pAd.CMV.NLS-CRE was verified by sequencing.

To produce the recombinant adenovirus carrying 10 the cre transgene, the pAd.CMV.NLS-CRE recombinant vector was co-transfected with the Ad dl327 backbone into 293 cells. Ten days later, 15 plaques were picked up and 5 of them were expanded on 293 cells. Viruses were screened for their recombinase activity by assessing 15 their ability to remove a spacer positioned between the CAG promoter (beta-actin) and the bacterial LacZ coding sequence using an adenoviral construct described in Y. Kanegae et al, Nucl. Acids Res., 23:3816-3821 (1995). Two viruses tested positive for beta-galactosidase 20 activity, indicating cre recombinase activity. As desired, these recombinant viruses may be purified by two rounds of plaque purification.

Example 2 - Construction of Ad.sp.Rep/Cap

An exemplary recombinant adenovirus containing 25 the AAV rep and cap genes may be produced as follows.

An AAV P5 promoter was obtained from the 121 bp XbaI-BamHI fragment from plasmid psub201, which contains the entire AAV2 genome [R.J. Samulski et al, J. Virol., 61:3096-3101 (1987)] by PCR using the following primer 30 pairs:

XbaI ITR rightward: SEQ ID NO:2:
GGCCTCTAGATGGAGGGTGGAGTCGTGAC;

BamP5 rightward: SEQ ID NO:3:
GGCCGGATCCAACCGCGCAGCCGCCATGCCG;

Bam P5 leftward: SEQ ID NO:4:

GGCCGGATCCAAACCTCCGCTTCAAAAT;

SacI leftward: SEQ ID NO:5:

GGCCGAGCTCAGGCTGGGTTTGGGGAGCA.

5 A 5' portion of the Rep/Cap gene was similarly excised via PCR from a BamI-SacI fragment (504 bp) obtained from psub201. The BamHI PCR primer creates a unique site between the rep mRNA and the first rep ATG.

10 The P5 promoter and the Rep/Cap gene fragment were subcloned into the XbaI-SacI sites of the pSP72 vector (Promega), resulting in P5.Rep/Cap. The spacer DNA, a 1300 bp fragment flanked by loxP sites, was obtained from the plasmid pMA19 [M. Anton and F. Graham, *J. Virol.*, 69:4600-4606 (1995)] following digestion with 15 BamHI. This spacer DNA was cloned into the unique BamHI site of the P5.Rep/Cap construct, resulting in the P5.Spacer.Rep/Cap construct.

20 The complete fragment containing the P5 promoter, the spacer and the rep/cap genes was obtained by subcloning the 3' portion of the Rep/Cap gene (SacI/blunt ended fragment, 3680 bp) into the SacI-EcoRV sites of the P5.Spacer.Rep/Cap plasmid. The 3' portion of the Rep/cap gene was isolated from the SSV9 plasmid (which contains a complete wild-type AAV genome) as a 25 SacI-blunt ended fragment. This involved digesting SSV9 with XbaI, filling the XbaI site with Klenow and liberating the fragment by digesting with SacI.

30 The complete fragment containing the P5 promoter, the spacer and the rep/cap sequence was subcloned into the BglII site of the pAd.link vector. This was accomplished by adding a BglII linker at the 5' end of the P5.Spacer.Rep/Cap plasmid construct and using the BglII site located at the 3' end of the multiple cloning site of pSP72.

The resulting plasmid (11250 bp) contains Ad5 map units (mu) 0-1, the P5 promoter, the spacer sequence flanked by loxP sites, rep/cap, and Ad5 mu 9-16. This plasmid is termed pAd.P5.spacer.Rep/Cap [Fig. 4].

To produce recombinant adenovirus capable of expressing rep and cap, pAd.P5.spacer.Rep/Cap was first used to transform a cre-expressing bacterial strain *E. coli* strain BNN132 (ATCC Accession No. 47059) in order to determine whether the spacer could be removed after recombination between the loxP sites (catalyzed by the cre recombinase). Analysis on agarose gels of the plasmid DNA isolated from several transformed colonies showed that, indeed, most of the constructs analyzed lost the spacer following transformation (data not shown).

The plasmid P5.spacer.Rep/Cap was also co-transfected with the Ad dl327 backbone in HEK 293 cells. Ten days later, 20 plaques were picked up and expanded. The structure of the viruses was analyzed by Southern blot using the complete AAV genome and the 1300 bp DNA spacer as probes. One plaque (P3) showed the expected band pattern after digestion with the restriction enzyme BamHI (data not shown).

Similar constructs may be made using other suitable spacers. For example, a 1600 bp spacer was derived from plasmid phGFP-S65T plasmid (Clontech) which contains the humanized GFP gene. phGFP-S65T was cut with the restriction enzymes HindIII and BamHI. After adding a BglII linker at the 5' end (BglII is compatible with BamHI), the 1.6 kb fragment was subcloned into the BamHI site of the flox vector [H. Gu et al, *Science*, 265:103-106 (1994)] in order to add a loxP site on each side of the fragment. The GFP DNA fragment flanked by loxP sites was subsequently cut with PvuI and SmaI and subcloned into the EcoRV site of the Bluescript II cloning vector.

(Stratagene). The resulting GFP spacer can be used to construct a P5.spacer.Rep/cap plasmid or adenovirus as described above.

Example 3 - Production of rAAV

5 The supernatant from several plaques (containing viruses) obtained from the study described in Example 2 was tested for the ability to produce AAV in a functional assay involving the adenovirus encoding the cre protein constructed as described in Example 1 above
10 and pAV.CMVLacZ.

The plasmid AV.CMVLacZ is a rAAV cassette in which rep and cap genes are replaced with a minigene expressing β -galactosidase from a CMV promoter. The linear arrangement of AV.CMVLacZ includes:

15 (a) the 5' AAV ITR (bp 1-173) obtained by PCR using pAV2 [C. A. Laughlin et al, Gene, 23: 65-73 (1983)] as template [nucleotide numbers 365-538 of SEQ ID NO:1];

20 (b) a CMV immediate early enhancer/promoter [Boshart et al, Cell, 41:521-530 (1985); nucleotide numbers 563-1157 of SEQ ID NO:1],

 (c) an SV40 intron (nucleotide numbers 1178-1179 of SEQ ID NO:1),

 (d) *E. coli* beta-galactosidase cDNA (nucleotide numbers 1356 - 4827 of SEQ ID NO:1),

25 (e) an SV40 polyadenylation signal (a 237 BamHI-BclI restriction fragment containing the cleavage/poly-A signals from both the early and late transcription units; nucleotide numbers 4839 - 5037 of SEQ ID NO:1) and

30 (f) 3'AAV ITR, obtained from pAV2 as a SnaBI-BglII fragment (nucleotide numbers 5053 - 5221 of SEQ ID NO:1).

The functional assay was performed by infecting 293 cells with the cre virus and the Rep/Cap virus

(multiplicity of infection (MOI) 10) followed by a transfection 2 hours later with 5 µg pAV.CMVLacZ. Forty-eight hours later, cells were harvested and freeze-thawed. One-fifth of the supernatant (containing rAAV) 5 was used to infect 293 cells. Twenty-four hours later an X-gal assay was performed.

Viruses from plaque #3 yielded positive for beta-galactosidase transduction in this assay.

Supernatant from plaque #3 was used in a second round of 10 purification (plaque amplification). Twenty plaques were picked up and expanded.

Example 4 - Production of Cre Expressing Cell Line

A plasmid vector, pG.CMV.nls.cre was constructed as follows for use in transfecting 293 cells. 15 The nls-Cre cDNA was isolated from the plasmid pexCANCRE (Kanegae, cited above) as described in Example 1 above. The nls-Cre fragment was then subcloned into the XbaI sites of vector pG downstream of a CMV promoter. This plasmid vector is illustrated in Figure 3 and contains a 20 human growth hormone (hGH) termination sequence, an SV40 ori signal, a neomycin resistance marker, an SV40 polyadenylation site, an ampicillin marker, on a backbone of pUC19.

This plasmid was transfected into 293 cells 25 using conventional techniques. Cells were selected in the presence of G-418 for neomycin resistance. Cells were identified by infecting them at different MOI (1 to 100) with Ad.CAG.Sp.LacZ, an adenovirus containing the bacterial LacZ coding sequence separated from its beta-actin (CAG) promoter by a neomycin spacer DNA flanked by 30 two loxP sites followed by the bacterial LacZ gene. Cells were selected on the basis of their ability to remove the spacer fragment inducing the expression of the LacZ gene. After X-gal staining, six cell lines were

found to be positive. DNA from these infected cells was isolated and analyzed by Southern blot using the spacer DNA (NEO) as a probe. Results shown in Fig. 6A, with reference to Table 1, and Figs. 6B - 6D indicate that 5 cell line #2 can remove the DNA spacer with much more efficacy than the other 293/cre cell lines analyzed.

Table 1

		<u>NEO Probe</u>	
		<u>Without Recombination</u>	<u>With Recombination</u>
10		6200	6200
15			5200

Example 5 - Generation of the Ad.GFP Rep/Cap

As described in Example 2 for the construction of the Ad.Sp.Rep/Cap virus, the link plasmid containing the P5 promoter, the GFP spacer flanked by two loxP sites 20 and the Rep and Cap coding sequences was co-transfected with the Ad dl327 backbone into HEK 293 cells. Ten days later, 20 plaques were picked up and expanded. During this expansion, the monolayer of HEK 293 cells were screened for the expression of GFP by microscopic 25 analysis using a mercury lamp with a 470-490 nm band-pass excitation filter (Nikon). One of the monolayers (from plaque #13) showed a region positive for the expression of GFP. This region was further expanded and purified by two other rounds of plaque purification. The presence of 30 the Ad.GFP.Rep/Cap virus was monitored by the expression of GFP, as described, and/or by the expression of the Rep and Cap proteins by Western blot analysis using specific monoclonal antibodies (American Research Products, Inc.). One cell lysate (from one purified plaque) containing the 35 Ad.GFP rep/cap was used in order to infect 293 cells

(adenovirus preparation with 40 x 150 mm dishes of HEK 293 cells). A total of 6.86×10^{13} particles/ml were obtained after purification. This virus is currently being tested for the production of rAAV, as described in
5 Example 3.

Example 6 - Construction of the Ad.TRE.CMV.GFP.Rep/Cap

Fig. 7 shows the final structure of the Ad.TRE.CMV.Rep/Cap virus. The AAV P5 promoter was replaced by the tetracycline (Tet) inducible promoter (Clontech). This promoter contains the tetracycline responsive elements (TRE) followed by the CMV minimal promoter without the CMV enhancer. This promoter is inducible in the presence of the antibiotic doxycycline (Sigma) in the 293/Tet-On cell line (Clontech) which contains a stable gene expressing the rTetR (reverse Tet repressor) fused to the GP16 transcriptional activation domain. The objective here is to construct a double inducible expression system in order to limit the expression of the cytotoxic Rep gene products. In order to fully induce the expression of the Rep and Cap genes, the virus must be in the presence of 1- the cre recombinase (in order to delete the GFP spacer as described previously) and 2- the Tet-On inducible factor doxycycline (DOX).

The link plasmid containing the construct described above was used to transfect HEK 293 cells in the presence or the absence of DOX and/or the cre recombinase (from the adenovirus expressing nls-cre). Proteins from cell homogenates were analyzed by Western blot using the Rep antibodies. Rep proteins are fully induced only in the presence of DOX and the cre recombinase.

In order to construct pAd.TRE.CMV.link.1, the pTRE plasmid (Clontech) was cut with the restriction

endonucleases *Xho* and *EcoRI* to isolate the TRE and the minimal CMV promoter. The *Xho* and *EcoRI* sites were filled with Klenow and the 448 bp fragment was inserted into the *EcoRV* site of the pAdlink.1 plasmid. The 5 GFP.Rep/Cap fragment was subsequently cut with *ClaI* and *BglIII* and inserted into the pAd.TRE.CMV.link.1 cut with *ClaI* and *BamHI*.

This link recombinant plasmid was co-transfected with the Ad dl327 backbone in HEK 293 cells. 10 Ten days later, 20 plaques were picked up and expanded. These plaques are currently being analyzed for the expression of GFP and the Rep and Cap proteins. Two adenoviruses expressing large amounts of rep proteins were identified. These viruses are currently being 15 purified and studied.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to 20 the processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania
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(ii) TITLE OF INVENTION: Methods using Cre-Lox for Production of Recombinant Adeno-Associated Viruses

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(v) COMPUTER READABLE FORM:

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 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10398 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCTGCTA GCATCATCAA TAATATACCT TATTTGGAT TGAAGCCAAT ATGATAATGA

60

GGGGGTGGAG TTTGTGACGT GCGCGGGGGC GTGGGAACGG GGCGGGTGAC GTAGTAGTGT

120

GGCGGAAGTG	TGATGTTGCA	AGTGTGGCGG	AACACATGTA	AGCGACGGAT	GTGGCAAAAG	180
TGACGTTTT	GGTGTGCC	GGTGTACACA	GGAAAGTGACA	ATTTTCGCGC	GGTTTTAGGC	240
GGATGTTGTA	GTAAATTG	GCGTAACCGA	GTAAAGATTTG	GCCATTTCG	CGGGAAAACT	300
GAATAAGAGG	AACTGAAATC	TGAATAATT	TGTGTTACTC	ATAGCGCGTA	ATATTTGTCT	360
AGGGAGATCT	GCTGCGCGCT	CGCTCGCTCA	CTGAGGCCGC	CCGGGCAAAG	CCCGGCGTC	420
GGCCGACCTT	TGGTCGCCCG	GCCTCAGTGA	CCGAGCGAGC	GCGCAGAGAG	GGAGTGGCCA	480
ACTCCATCAC	TAGGGTTCC	TTGTAGTTAA	TGATTAACCC	GCCATGCTAC	TTATCTACAA	540
TCGAGCTTG	CATGCCCTGCA	GGTCGTTACA	TAACTTACGG	TAATGGCCC	GCCTGGCTGA	600
CCGCCAACG	ACCCCCGCC	ATTGACGTCA	ATAATGACGT	ATGTTCCCAT	AGTAACGCCA	660
ATAGGGACTT	TCCATTGACG	TCAATGGGTG	GAGTATTAC	GGTAAACTGC	CCACTTGGCA	720
GTACATCAAG	TGTATCATAT	GCCAAGTACG	CCCCCTATTG	ACGTCAATGA	CGGTAAATGG	780
CCCGCCTGGC	ATTATGCCCA	GTACATGACC	TTATGGGACT	TTCCCTACTTG	GCAGTACATC	840
TACGTATTAG	TCATCGCTAT	TACCATGGTG	ATGCGTTTT	GGCAGTACAT	CAATGGCGT	900
GGATAGCGGT	TTGACTCACG	GGGATTTCCA	AGTCTCCACC	CCATTGACGT	CAATGGGAGT	960
TTGTTTGGC	ACCAAAATCA	ACGGGACTTT	CCAAAATGTC	GTAACAACTC	CGCCCCATTG	1020
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AACCGTCAGA	TGGCCTGGAG	ACGCCATCCA	CGCTGTTTG	ACCTCCATAG	AAGACACCCG	1140
GACCGATCCA	GCCTCCGGAC	TCTAGAGGAT	CCGGTACTCG	AGGAACTGAA	AAACCCAGAAA	1200
GTTAACTGGT	AAGTTTAGTC	TTTTGTCTT	TTATTTCAAG	TCCCGGATCC	GGTGGTGGTG	1260
CAAATCAAAG	AACTGCTCCT	CAGTGGATGT	TGCCTTACT	TCTAGGCCTG	TACGGAAGTG	1320
TTACTTCTGC	TCTAAAAGCT	GCGGAATTGT	ACCCGGGCC	GCAATTCCCG	GGGATCGAAA	1380
GACCTGCTA	AAGCAAAAAA	GAAGTCACCA	TGTCGTTAC	TTGACCAAC	AAGAACGTGA	1440
TTTCGTTGC	CGGTCTGGGA	GGCATTGGTC	TGGACACCAAG	CAAGGAGCTG	CTCAAGCGCG	1500
ATCCCCTCGT	TTTACAACGT	CGTACTGGG	AAAACCTGG	CGTTACCCAA	CTTAATCGCC	1560
TTGCAGCACA	TCCCCCTTC	GCCAGCTGGC	GTAATAGCGA	AGAGGCCGC	ACCGATCGCC	1620
CTTCCCAACA	GTTGCGCAGC	CTGAATGGCG	AAATGGCGCTT	TGCTGGTTT	CCGGCACCAAG	1680
AAGCGGTGCC	GGAAAGCTGG	CTGGAGTGCG	ATCTTCTGA	GGCCGATACT	GTCGTGTC	1740
CCTCAAACG	GCAGATGCAC	GGTTACGATG	CGCCCATCTA	CACCAACGTA	ACCTATCCCA	1800
TTACGGTCAA	TCCGCCGTT	GTTCCCACGG	AGAATCCGAC	GGGTTGTTAC	TCGCTCACAT	1860
TTAATGTTGA	TGAAAGCTGG	CTACAGGAAG	GCCAGACGCG	AATTATTTT	GATGGCGTTA	1920
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TGCCGTCTGA	ATTTGACCTG	AGCGCATTTC	TACGGCCGG	AGAAAACCGC	CTCGCGGTGA	2040
TGGTGCTGCG	TTGGAGTGAC	GGCAGTTATC	TGAAAGATCA	GGATATGTGG	CGGATGAGCG	2100
GCATTTCCG	TGACGTCTCG	TTGCTGCATA	AACCGACTAC	ACAAATCAGC	GATTTCATG	2160
TTGCCACTCG	CTTTAATGAT	GATTTCAGCC	GCGCTGTACT	GGAGGCTGAA	GTTCAGATGT	2220
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AAGGCGCCG	AGCCGACACC	ACGGCCACCG	ATATTATTTG	CCCGATGTAC	GCGCGCGTGG	3000
ATGAAGACCA	GCCCTTCCC	GCTGTGCCA	AATGGTCCAT	AAAAAAATGG	CTTTCGCTAC	3060
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TCTTGCCTG	CCGCACGCCG	CATCCAGCCG	TGACGGAAGC	AAAACACCAG	CAGCAGTTT	3360
TCCAGTTCCG	TTTATCCGGG	CAAACCATCG	AAGTGACCGAG	CGAATACCTG	TTCCGTCATA	3420
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AA GTGCCTCT	GGATGTCGCT	CCACAAGGTA	AACAGTTGAT	TGA ACTGCCT	GA ACTACCGC	3540
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GTGTGACGCT	CCCCGCCGCG	TCCCACGCCA	TCCCGCATCT	GACCACCAGC	GAAATGGATT	3720
TTTGCATCGA	GCTGGTAAT	AAGCGTTGGC	AATTTAACCG	CCAGTCAGGC	TTTCTTCAC	3780
AGATGTGGAT	TGGCGATAAA	AAACAAC TGC	TGACGCCGCT	GCGCGATCAG	TTCACCCGTG	3840

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TCGAACGCTG	GAAGGGGGCG	GGCCATTACC	AGGCCGAAGC	ACCGTTGTTG	CAGTGCACGG	3960
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CGGGCAGGCC	ATGTCTGCC	GTATTCGCG	TAAGGAAATC	CATTATGTAC	TATTTAAAAA	4620
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ACTTCCCATT	TTTCCCATT	TGGCTACATG	ACATCAACCA	TATCAGCAA	AGTGTACCGG	4740
GTATTATTTT	TGCCGCTATT	TCTCTGTTCT	CGCTATTATT	CCAACCGCTG	TTTGGTCTGC	4800
TTTCTGACAA	ACTCGGCCTC	GAECTCTAGGC	GGCCGGGGGG	ATCCAGACAT	GATAAGATAC	4860
ATTGATGAGT	TTGGACAAAC	CACAACTAGA	ATGCAGTGAA	AAAAATGCTT	TATTTGTGAA	4920
ATTGTGATG	CTATTGCTT	ATTGTAAACC	ATTATAAGCT	GCAATAAAC	AGTTAACAAAC	4980
AACAATTGCA	TTCATTTAT	GTTTCAGGTT	CAGGGGGAGG	TGTGGGAGGT	TTTTTGGAT	5040
CCTCTAGAGT	CGAGTAGATA	AGTAGCATGG	CGGGTTAAC	ATTAACATACA	AGGAACCCCT	5100
AGTGATGGAG	TTGGCCACTC	CCTCTCTGCG	CGCTCGCTCG	CTCACTGAGG	CCGGGCGGACC	5160
AAAGGTCGCC	CGACGCCCGG	GCTTGCCCG	GGCGGCCTCA	GTGAGCGAGC	GAGCGCGCAG	5220
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GGCGGTAAAC	ATATTAGGAA	CCAGCCTGTG	ATGCTGGATG	TGACCGAGGA	GCTGAGGCC	5340
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TGAGGTACTG	AAATGTGTGG	GGGTGGCTTA	AGGGTGGAA	AGAATATATA	AGGTGGGGT	5460
CTTATGTACT	TTTGTATCTG	TTTGCAGCA	GCCGCCGCCG	CCATGAGCAC	CAACTCGTT	5520
GATGGAAGCA	TTGTGAGCTC	ATATTGACA	ACCGCGCATGC	CCCCATGGGC	CGGGGTGCGT	5580
CAGAATGTGA	TGGGCTCCAG	CATTGATGGT	CGCCCCGTCC	TGCCCCGCAA	CTCTACTACC	5640
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GCCGCTGCAG	CCACCGCCCG	CGGGATTGTG	ACTGACTTTG	CTTTCCTGAG	CCCGCTTGCA	5760
AGCAGTGCAG	CTTCCCCTTC	ATCCGCCGC	GATGACAAGT	TGACGGCTCT	TTTGGCACAA	5820
TTGGATTCTT	TGACCCGGGA	ACTTAATGTC	GTTCCTCAGC	AGCTGTTGGA	TCTGCGCCAG	5880
CAGGTTCTG	CCCTGAAGGC	TTCCCTCCCCT	CCCAATGCCG	TTTAAACAT	AAATAAAAAA	5940
CCAGACTCTG	TTTGGATTTC	GATCAAGCAA	GTGTCTTGCT	GTCTTTATTT	AGGGGTTTTG	6000
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GGCAGGCCCT	TGGTGTAACT	GTTCACAAAG	CGGTTAACGCT	GGGATGGGTG	CATACGTGGG	6300
GATATGAGAT	GCATCTTGGA	CTGTATTTTT	AGGTTGGCTA	TGTTCCCAGC	CATATCCCTC	6360
CGGGGATTCA	TGTTGTGCAG	AACCACCAGC	ACAGTGTATC	CGGTGCACCT	GGGAAATTG	6420
TCATGTAGCT	TAGAAGGAAA	TGCGTGGAAAG	AACTTGGAGA	CGCCCTTG TG	ACCTCCAAGA	6480
TTTCCATGC	ATTCGTCCAT	AATGATGGCA	ATGGGCCCAC	GGCGGGCGGC	CTGGGCGAAG	6540
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TTTACAAAGC	GCAGGGCGGAG	GGTGCCAGAC	TGCGGTATAA	TGGTTCCATC	CGGCCAGGG	6660
GGCTAGTTAC	CCTCACAGAT	TTGCATTTC	CACGCTTTGA	GTTCAGATGG	GGGGATCATG	6720
TCTACCTGCC	GGGCGATGAA	AAAAACGGTT	TCCGGGGTAG	GGGAGATCAG	CTGGGAAGAA	6780
AGCAGGTTCC	TGAGCAGCTG	CGACTTACCG	CAGCCGGTGG	GCCCCTAAT	CACACCTATT	6840
ACCGGGTGCA	ACTGGTAGTT	AAGAGAGCTG	CAGCTGCCGT	CATCCCTGAG	CAGGGGGGCC	6900
ACTTCGTTAA	GCATGTCCCT	GAETCGCATG	TTTCCCTGA	CCAAATCCGC	CAGAAGGCGC	6960
TCGCCGCCCA	GCGATAGCAG	TTCTTGCAG	GAAGCAAAGT	TTTCAACGG	TTGAGACCG	7020
TCCGCCGTAG	GCATGCTTT	GAGCGTTGA	CCAAGCAGTT	CCAGGCGGTC	CCACAGCTCG	7080
GTACACCTGCT	CTACGGCATC	TCGATCCAGC	ATATCTCCTC	GTTCGCGGG	TTGGGGCGGC	7140
TTTCGCTGTA	CGGCAGTAGT	CGGTGCTCGT	CCAGACGGGC	CAGGGTCATG	TCTTCCACG	7200
GGCGCAGGGT	CCTCGTCAGC	GTAGTCTGGG	TCACGGTGAA	GGGGTCCGCT	CGGGGCTGCC	7260
CGCTGGCCAG	GGTGGCGTTG	AGGCTGGTCC	TGCTGGTGCT	GAAGCGCTGC	CGGTCTTCGC	7320
CCTGCCGCTC	GGCCAGGTAG	CATTGACCA	TGGTGTCTATA	GTCCAGCCCC	TCCGCCGCGT	7380
GGCCCTTGGC	GCGCAGCTTG	CCCTTGGAGG	AGGCCCGCAG	CGAGGGGCAG	TGCAGACTTT	7440
TGAGGGCGTA	GAGCTTGGGC	GCGAGAAATA	CCGATTCCGG	GGAGTAGGCA	TCCGCCGCC	7500
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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCCGGATCC AACGCGCAGC CGCCATGCCG

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32

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

- (ii) MOLECULE TYPE: other nucleic acid

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCCGGATCC CAAACCTCCC GCTTCAAAAT

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(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

- (ii) MOLECULE TYPE: other nucleic acid

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCCGAGCTC AGGCTGGGTT TTGGGGAGCA

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WHAT IS CLAIMED IS:

1. A method for production of recombinant adeno-associated virus (AAV) comprising culturing a host cell comprising and capable of expressing

(a) a cre transgene, which permits splicing out of the rep and cap gene inhibitory sequences that when removed lead to activation of rep and cap;

(b) AAV rep and cap genes having a spacer 5' thereto, said spacer flanked by lox sites;

(c) a minigene comprising a therapeutic transgene flanked by AAV inverse terminal repeats (ITRs); in the presence of sufficient helper virus functions, wherein a recombinant AAV capable of expressing said transgene is produced.

2. The method according to claim 1 further comprising:

(a) introducing into a selected host cell i. a first vector comprising a cre gene under control of sequences which permit expression of cre recombinase;

ii. a second vector comprising from 5' to 3', a selected promoter, a spacer sequence flanked by loxP sites, an AAV rep gene and an AAV cap gene;

iii. a third vector comprising a minigene consisting essentially of, from 5' to 3', a 5' AAV inverse terminal repeat (ITR), a selected promoter, a selected transgene and 3' AAV ITR;

(b) culturing the host cell under conditions which permit expression of the cre recombinase; and

(c) recovering recombinant AAV capable of expressing the product of said transgene.

3. The method according to claim 1 wherein at least one of said vectors is a recombinant adenovirus and the host cell is a 293 cell.

4. The method according to claim 1 wherein the first vector is a recombinant adenovirus and the sequences which permit expression comprise a cytomegalovirus promoter, the vector further comprising a nuclear localization signal operably linked to the cre gene.

5. The method according to claim 1 wherein the second vector is a recombinant adenovirus and the selected promoter comprises AAV P5.

6. The method according to claim 5 wherein the spacer sequence is selected from the group consisting of:

- (a) a 1300 bp fragment containing translational start and stop sequences;
- (b) a 1600 bp fragment containing the GFP cDNA, an intron and a polyadenylation signal; and
- (c) a 1000 bp fragment containing the neomycin coding sequence and a polyadenylation signal..

7. A method for production of recombinant adeno-associated virus (AAV) comprising:

- (a) providing a host cell expressing cre;
- (b) introducing into said host cell a first vector comprising from 5' to 3', a selected promoter, a spacer sequence flanked by loxP sites, and AAV rep and cap genes; and

a second vector comprising from 5' to 3', a minigene consisting essentially of 5' AAV inverse terminal repeat (ITR), a selected promoter, a selected transgene, and a 3' AAV ITR;

(c) culturing the host cell under conditions which permit expression of the cre recombinase and replication and packaging of a recombinant AAV; and

(d) recovering the recombinant AAV capable of expressing the product of the transgene.

8. The method according to claim 7 wherein the first and second vectors are recombinant adenoviruses.

9. The method according to claim 8 wherein the spacer sequence is selected from the group consisting of:

(a) a 1300 bp fragment containing translational start and stop sequences;

(b) a 1600 bp fragment containing the GFP cDNA, an intron and a polyadenylation signal; and

(c) a 1000 bp fragment containing the neomycin coding sequence and a polyadenylation signal.

10. A recombinant AAV produced according to the method of any one of claims 1 - 9.

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GFP SPACER

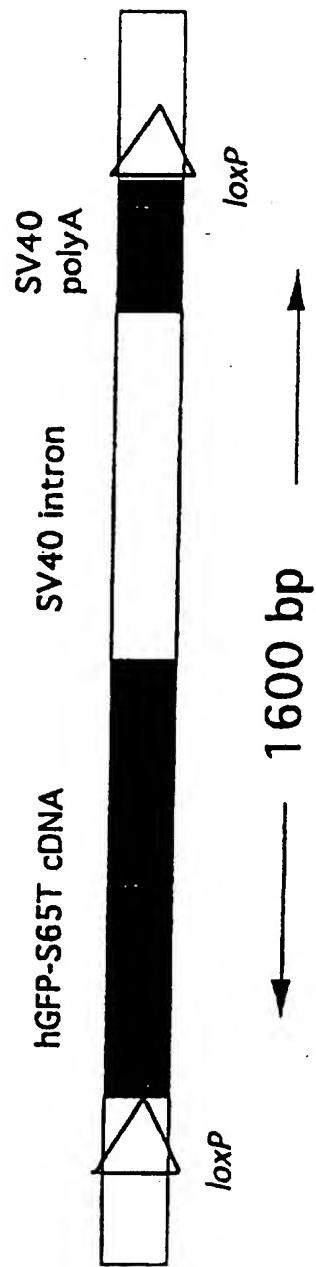


Fig. 1

NEO SPACER

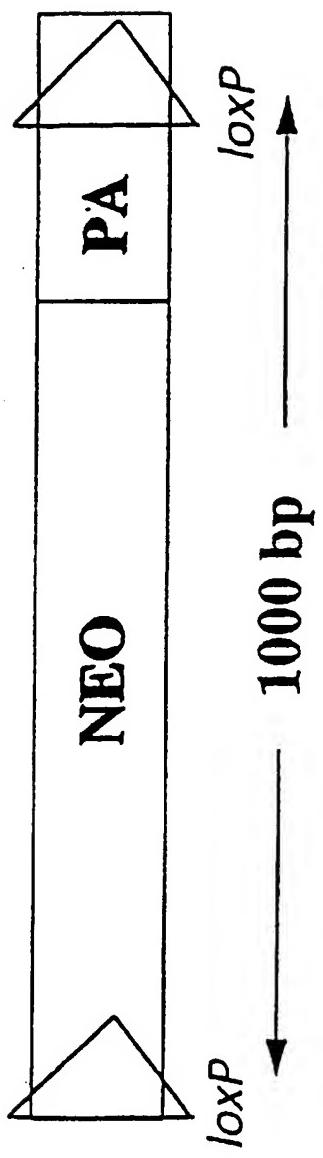


Fig. 2

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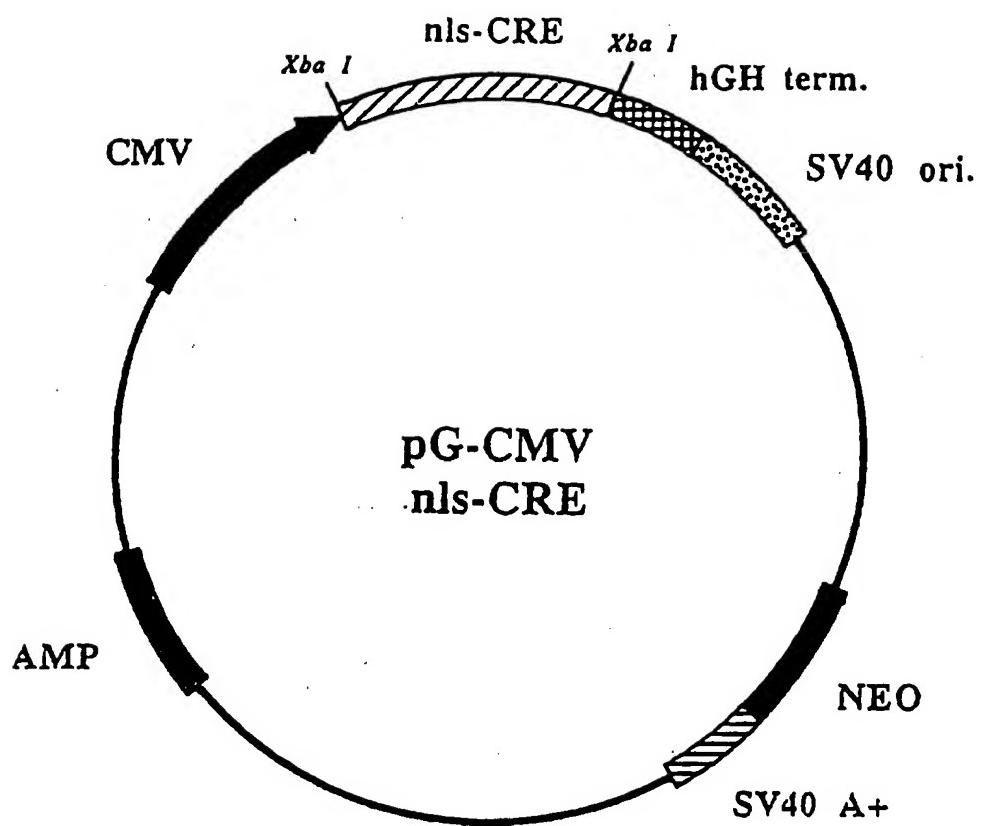


Fig. 3

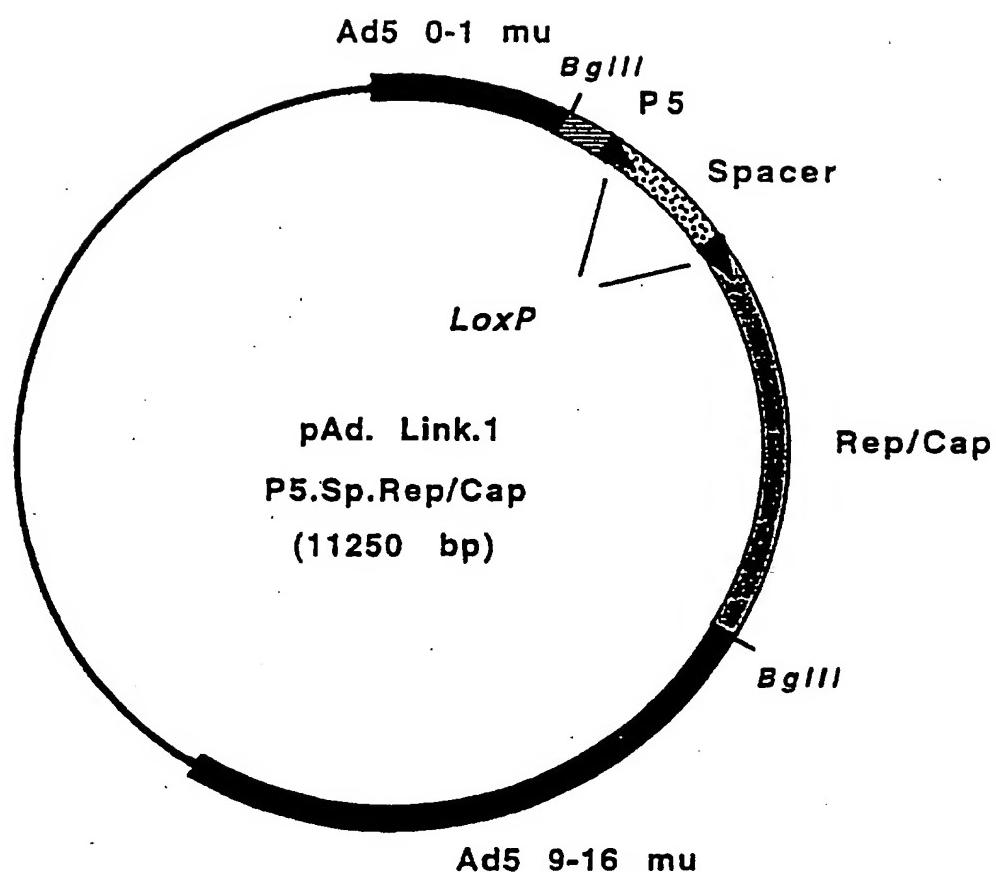
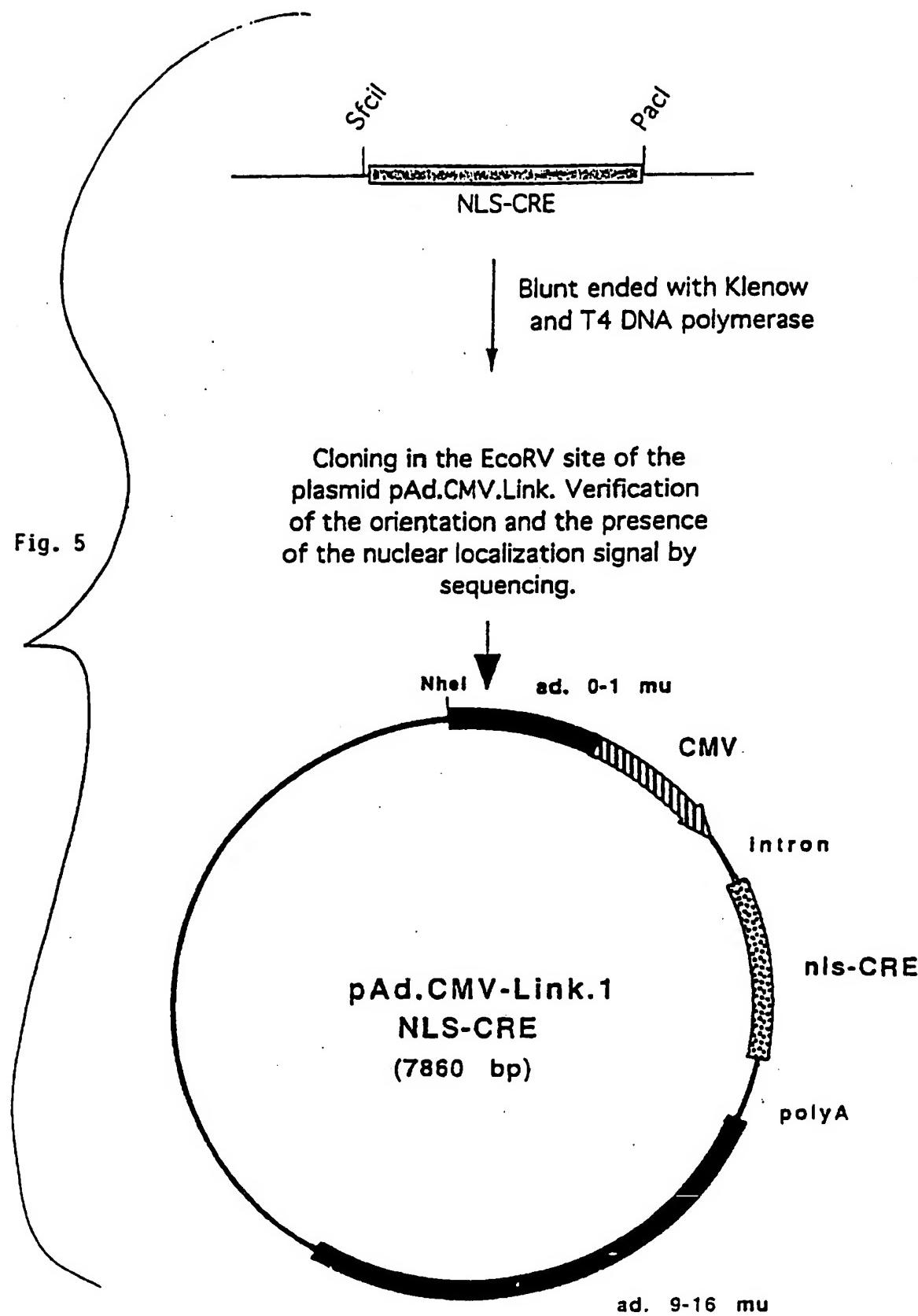
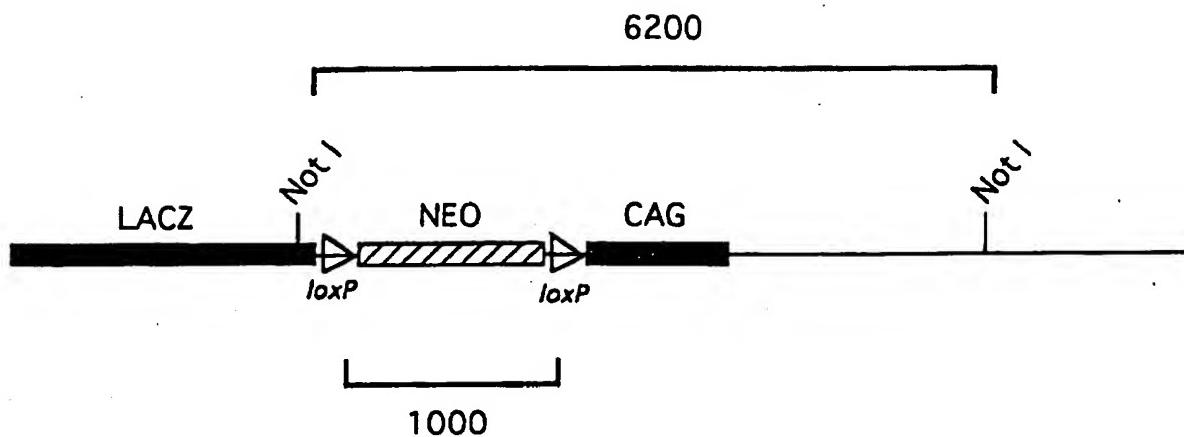


Fig. 4

Construction of the Ad. CMV. NLS-CRE

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Fig. 6A



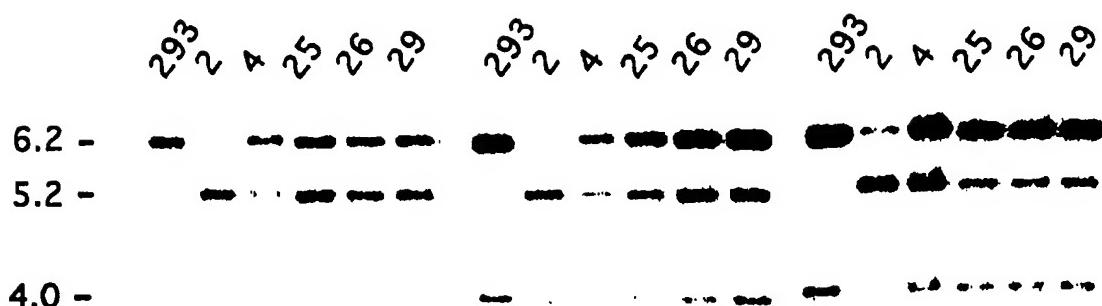
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Fig. 6B

Fig. 6C

Fig. 6D

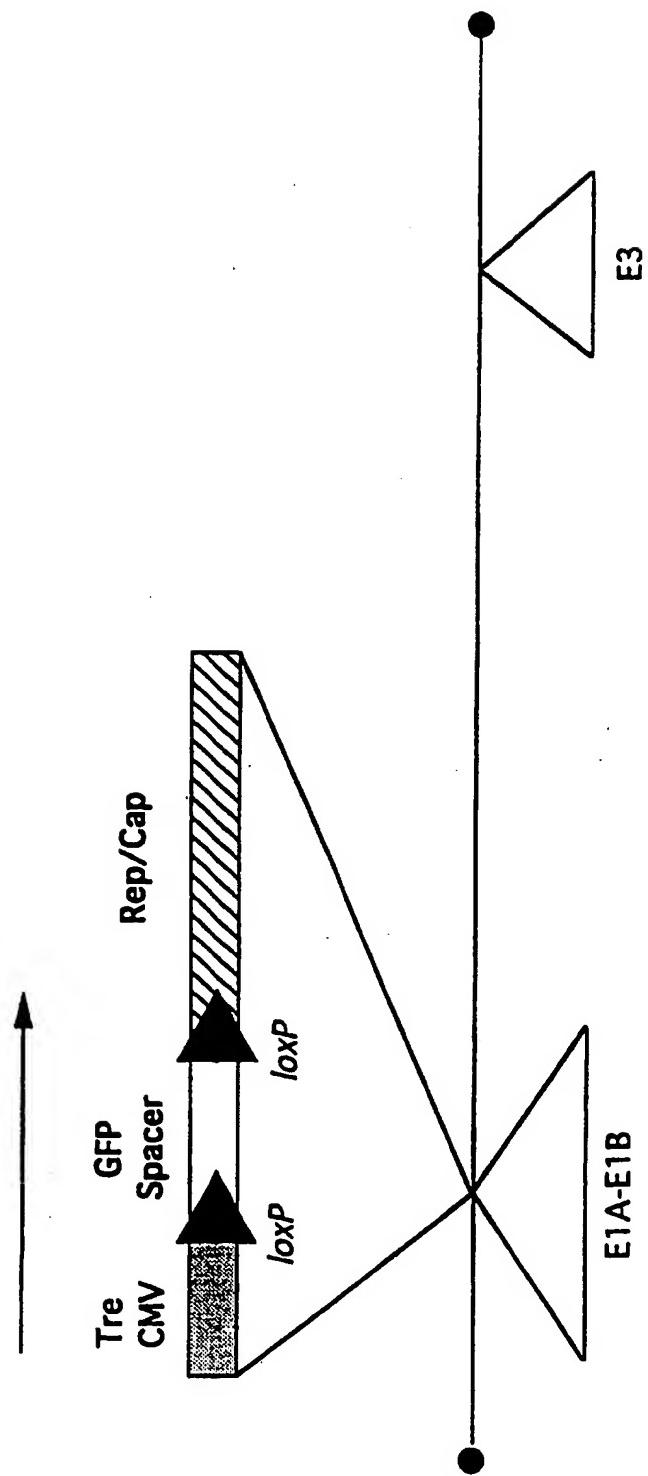


FIG. 7

INTERNATIONAL SEARCH REPORT

Interr. Application No
PCT/US 97/15691

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C07K14/015 C12N9/00 C12N9/52 C12N15/35
C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 17947 A (TARGETED GENETICS CORP ;ALLEN JAMES M (US)) 13 June 1996 see the whole document ---	1-10
Y	ANTON M ET AL: "SITE-SPECIFIC RECOMBINATION MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING THE CRE RECOMBINASE PROTEIN: A MOLECULAR SWITCH FOR CONTROL OF GENE EXPRESSION" JOURNAL OF VIROLOGY, vol. 69, no. 8, August 1995, pages 4600-4606, XP002011775 see page 4602, column 1, line 4 - page 4604, column 2, line 41; figures 1,4A,5A ---	1-10 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- "&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
13 February 1998	23/02/1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Chambonnet, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/15691

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation or document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	KANEGAE Y ET AL: "EFFICIENT GENE ACTIVATION IN MAMMALIAN CELLS BY USING RECOMBINANT ADENOVIRUS EXPRESSING SITE-SPECIFIC CRE RECOMBINASE" NUCLEIC ACIDS RESEARCH, vol. 23, no. 19, 11 October 1995, pages 3816-3821, XP002011774 see page 3818, column 1, paragraph 4 - page 3819, column 1, paragraph 2; figures 1,3 see page 3821, column 1, paragraph 5 ---	1-3,6-10
Y	WANG P ET AL: "HIGH FREQUENCY RECOMBINATION BETWEEN LOXP SITES IN HUMAN CHROMOSOMES MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING CRE RECOMBINASE" SOMATIC CELL AND MOLECULAR GENETICS, vol. 21, no. 6, November 1995, pages 429-441, XP000617918 see page 439, column 2, paragraph 2 - page 440, column 1, paragraph 2 ---	1-4
P,X	KANEGAE Y ET AL: "Efficient gene activation system on mammalian cell chromosomes using recombinant adenovirus producing Cre recombinase" GENE, vol. 181, no. 1-2, 28 November 1996, page 207-212 XP004071882 see page 212, column 1, line 1 - line 10; figure 1 ---	1-10
Y	WO 95 13392 A (OHIO MED COLLEGE ; TARGETED GENETICS CORP (US); TREMPE JAMES P (US)) 18 May 1995 see the whole document ---	1-10
Y	WO 95 13365 A (TARGETED GENETICS CORP ; UNIV JOHNS HOPKINS (US); FLOTTE TERENCE R) 18 May 1995 see the whole document ---	1-10
Y,P	WO 97 06272 A (AVIGEN INC) 20 February 1997 see page 6, line 28 - page 7, line 4 ---	1-5,7,8, 10
A	SNAITH M R ET AL: "Multiple cloning sites carrying loxP and FRT recognition sites for the Cre and Flp site-specific recombinases" GENE, vol. 166, no. 1, 1 January 1995, page 173-174 XP004043130 ---	1

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/15691

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	BEATON A ET AL: "EXPRESSION FROM THE ADENO-ASSOCIATED VIRUS P5 AND P19 PROMOTERS IS NEGATIVELY REGULATED IN TRANS BY THE REP PROTEIN" JOURNAL OF VIROLOGY, vol. 63, no. 10, October 1989, pages 4450-4454, XP000609489 -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/15691

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9513365 A	18-05-95	AU 1129395 A CA 2176117 A EP 0733103 A JP 9509564 T US 5658776 A	29-05-95 18-05-95 25-09-96 30-09-97 19-08-97
WO 9706272 A	20-02-97	US 5622856 A	22-04-97

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